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A Quantitative Assay for Intercellular Adhesion

(confluent cell monolayers/homologous and heterologous cell adhesions)

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ABSTRACT Intercellular adhesion is measured by a new method based on determination of the rates of attachment of single cells to confluent cell monolayers. The procedure is simple, rapid, and reproducible. Specific and nonspecific intercellular adhesions can be quantitated and distinguished from each other, and from the adhesion to glass (or plastic). The rate of adhesion of single cells to the monolayer is characteristic of more than 80% of the single-cell population. This method, therefore, provides a means for study of the molecular basis of intercellular adhesion.

Intercellular adhesion, both of homologous (specific) and heterologous (nonspecific) cells, is thought to be of fundamental importance in diverse physiological phenomena, including normal and abnormal development, metastasis, etc. While several methods have been developed to quantitate adhesion (1–5), it is doubtful that each method measures the same molecular event. Studies in this laboratory have concentrated on the earliest detectable process, the rates at which single cells adhere to each other or to aggregates. A rapid and reproducible quantitative assay involving large numbers of cells was developed with the Coulter electronic particle counter (6), but this method does not distinguish between specific and nonspecific adhesion in mixed populations. A collecting aggregate assay (7), on the other hand, can detect specific and nonspecific adhesion. However, the latter method is semiquantitative and the measured adhesive rates may not be characteristic of the whole cell population. The present paper describes a general method that permits swift and precise measurements of homologous and heterologous cell adhesions.

EXPERIMENTAL PROCEDURE

Materials. Cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, Inc.) supplemented with 10% calf serum (Gibco, Inc., heat-inactivated at 57° for 30 min), 100 units/ml of penicillin, and 100 μg/ml of streptomycin sulfate (Gibco, Inc.). Embryonic cells were cultured in the same basic medium with 10% heat-inactivated fetal-calf serum (Gibco, Inc.) in place of calf serum, and the addition of 1% Gibco 100x nonessential amino acids. These media are designated Medium A. For passage, the cell lines were washed with Ca^{++}–Mg^{++}-free phosphate-buffered saline (pH 7.4) (Gibco, Inc.) and dispersed with 0.25% Gibco trypsin solution; the trypsin solution was prepared in a glucose salts solution lacking Ca^{++} and Mg^{++} (Gibco "solution A"). Purified trypsin (3× crystallized) and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp.

The cells were maintained at 37° in a water-saturated atmosphere of 95% air–5% CO_2 to buffer the medium at pH 7.3. During adhesion experiments, the cells were maintained in serum-free medium containing Hank's salts, glucose, but without phenol red (Gibco, Inc.), 2% of 50× essential amino acids (Gibco, Inc.), and buffered with 0.01M HEPES (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid) (Calbiochem), pH 7.3 (Medium B).

Cells and Cell Lines. In the initial experiments, a cell line was used that grew to very high cell densities in order to cover the available (glass or plastic) substratum completely. To this end, BHK cells were passed repeatedly (more than 50 times) and were subsequently grown under starvation conditions; in two independent series of experiments, a BHK line (sBHK) was obtained that attained densities of 300,000 cells per cm^2. These cells were always seeded for adhesion experiments at this density; monolayers at this density were also used to prepare single-cell suspensions used for the adhesion studies.

A low-passage BHK cell (nBHK) was obtained from McPherson's laboratory (clone-13). This cell line attained a density of 70,000 cells per cm^2. Cells harvested at this density were used for preparation of both the monolayers and the single-cell suspensions.

A mouse teratoma cell line was derived by Dr. E. McGuire from strain T129 mice (8). Mouse kidney cells (MKA cells) were obtained from secondary cultures from strain A-J mice (Jackson Laboratories) and used after six passages.

Balb/c 3T3 cells (highly contact-inhibited, clone 3, designated 3T3) and SV40-transformed Balb/c 3T3 cells (clone 22, designated SV3T3) were obtained from Dr. S. Roth. Before use these cells were grown for a few passages in BHK medium containing 10% calf serum and 1% antibiotics. Neural retina cells were obtained from 8½-day-old, and liver and heart cells from 11½-day-old, White leghorn chick embryos. The tissues were dissociated by one (neural retina) or six (heart and liver) cycles of treatment with 0.25% trypsin in 10% (for neural retina) or 50% (for heart and liver) Medium A, and maintained in near confluent monolayers for 24 hr on Falcon culture dishes in Medium A. The cells were washed free of debris and erythrocytes, dispersed with the respective trypsin solutions described above, and reseeded at confluency in Linbro multiple-well tissue culture dishes (Linbro Chemical Co., New Haven, Conn.). These monolayers were maintained for 48 hr before use. For dissociation and dispersion of embryonic cells, 0.25% trypsin containing 0.5 mM EGTA proved equally useful.

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Preparation of Labeled Single Cells. All cells were labeled with [^{3}H]leucine (New England Nuclear Corp.; specific activity, 5 Ci/mmol) at 10–50 μCi/ml in Medium A; cells labeled with [^{3}H]uridine, [^{3}H]thymidine, [^{32}P]phosphate, or [^{14}C]acetate gave results essentially identical with those obtained with [^{3}H]leucine. Monolayers exposed to [^{3}H]leucine for the indicated times gave the following results: sBHK and teratoma cells, 15 hr, 0.6–1.6 cpm per cell; MKA, liver, heart, and neural retina, 40 hr, 0.1–0.5 cpm per cell. The [^{3}H]leucine was added to confluent monolayers; incorporation of the label was primarily due to protein turnover rather than cell division. More than 98% of the radioactivity in the cells was precipitable with cold 5% trichloroacetic acid. The monolayers were washed twice with phosphate buffered saline, and incubated in Medium A without [^{3}H]leucine for a minimum of 5 hr before preparation of the single-cell suspension; this method resulted in the loss of essentially all of the tritiated material soluble in the trichloroacetic acid test solution from the cells in 2 hr.

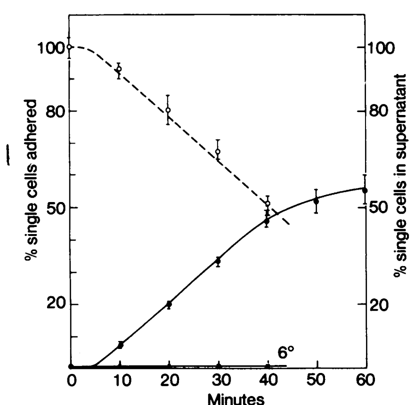

FIG. 1. Kinetics of cell adhesion. sBHK monolayers and sBHK single-cell suspensions were prepared as described in the text. The single cells were labeled with [^{3}H]leucine (0.7 cpm per cell), and 1.0-ml aliquots, containing 3 $\times$ 10^5 cells, were added to the monolayers (in Wheaton glass vials). Incubations were conducted at either 6° (⟵—) or 37° (●,O) for the indicated times. The supernatant fluid was removed from each vial and the monolayer was washed twice with 1-ml portions of Medium B. The washings and supernatant fluid were combined, and the cell density (O) was determined with a Coulter counter (6). Radioactivity in the monolayers was determined as described in the text after incubation at 37° (●——●) or 6° (⟵—). Extensive kinetic studies gave the following results: (*i*) The rate-limiting step under standard conditions was adhesion rather than sedimentation of the cells in the suspension. With 2-cm^2 monolayers, 10^5 cells were tested in volumes ranging from 0.1–2.0 ml. The adhesive rate (40.1 $\pm$ 1.8% cells adhered per 30 min) was constant from 0.1–0.8 ml. The lag period (about 5 min) was likewise unaffected. (*ii*) The labeled cells formed stable bonds with the monolayer; the maximal rate of detachment of adhered single cells was less than 3% of the adhesive rate. (*iii*) Cells in the monolayer did not exchange with cells in the suspension. Monolayers of radioactive cells (4.5 cm^2) lost less than 3% of the total label when incubated for 30 min with or without 10^6 unlabeled single cells. (*iv*) The cells attached at 40 min do not represent a subpopulation. Four sequential passages of the suspension from one monolayer to another resulted in adhesion of more than 83% of the single-cell population, with identical kinetics. (*v*) The declining adhesive rate is due in part to the reduction in single-cell number in the suspension (caused by adhesion) and to the diminishing adhesiveness shown by cells in the monolayer upon prolonged incubation in (serum-free) Medium B. It has been reported that serum-free medium causes the release of adhesive factors from cell monolayers (10).

Labeled single-cell suspensions were prepared by treatment of monolayers of cell lines with 0.25% Gibco trypsin solution (see *Materials*), 1 ml per 80 cm^2 of cells at 37°. The length of trypsinization varied with the cell type, being about 5 min for sBHK, 10 min for nBHK, heart, and neural retina, and 20

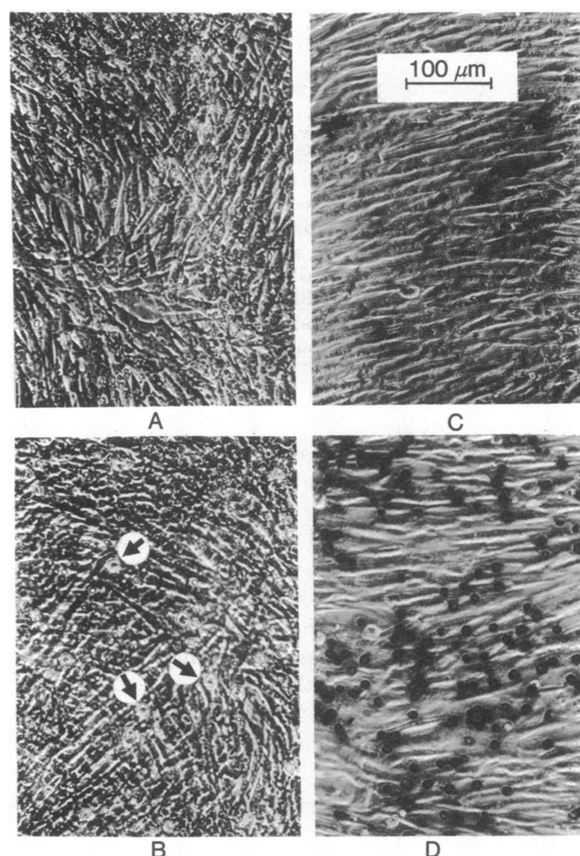

FIG. 2. Adhesion of sBHK cells to sBHK monolayers. Suspensions (0.5 ml) of single sBHK cells (0.9 cpm per cell; 10^5 cells per ml) were incubated with 2 cm^2 of sBHK monolayers on Linbro plates under standard conditions. Subsequently, the monolayers were incubated in Medium B containing 1% glutaraldehyde for 16 hr at 4°. Photographs were taken with a Wild phase-contrast microscope. (*A*) and (*B*) represent micrographs, while (*C*) and (*D*) are autoradiographs. (*A*) Control monolayer, not exposed to single cells. The small rounded bodies are granules within the monolayer. In most fields, no single, round cells were observed on top of the monolayer. (*A*) shows an exceptional field, containing a single cell (towards the center of the field) lying on the monolayer. (*B*) Monolayer incubated with single-cell suspension for 25 min. Numerous, single round cells lying on top of the monolayer are evident. The *arrows* point to three such cells. In (*C*) and (*D*), the monolayers were exposed to the single cells for 8 and 25 min, respectively, and after fixation, the monolayers were rinsed thoroughly in Medium B and incubated at 4° for 1 week with Kodak Nuclear Track emulsion NTB-2. All radioactive cells appear to be on top of the monolayers. No labeled cells (<0.2%) were found attached to rare gaps in the monolayer where plastic substratum is exposed. Labeled single cells are found over all parts of the monolayer, but are somewhat more numerous towards the center and over the peripheral parts of the monolayer (see *D*). Noncontiguous labeled cells are often located in strings following the long axis of the monolayer cells.

min for MKA, and teratoma cells. At this time, 5 ml of phosphate buffered saline per 80 cm^2 were added to the embryonic and the teratoma cells. The cells were dispersed by pipetting and incubated for an additional 5 min. A 3-fold volume (15 ml) of cold Medium A was added to halt trypsinization, the cells were centrifuged at 4° for 10 min at $100 \times g$, resuspended in 10 ml of cold Medium B, centrifuged at $200 \times g$ for 10 min at 4°, and finally resuspended in 10 ml of cold Medium B. After an aliquot was counted in a hemocytometer, the suspension was adjusted to the required concentration with Medium B at 37°. Teratoma cell suspensions were pipetted through a 27-gauge needle to obtain satisfactory single-cell preparations. (Those containing more than 10% of cells in aggregates were discarded.) About 4–8% of the total counts in the single-cell preparations did not sediment with the cells ($200 \times g$ for 10 min). With a second washing and centrifugation, the fraction declined slightly (to 3–6%) but could not be further reduced by subsequent washing. The "soluble" radioactivity probably reflects breakage and damage of the cells during resuspension, but does not represent a significant source of error in the adhesion assay. Under standard conditions the "soluble" radioactivity was taken up at negligible rates by the monolayer (<3% of the total cpm in this fraction per hr).

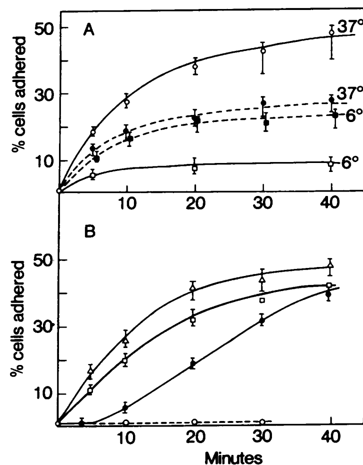

FIG. 3. Kinetics of sBHK cell adhesion to monolayers and to glass. (*A*) Attachment of cells to glass. One-milliliter aliquots of single sBHK cell suspension (30,000 cells per ml; 1.1 cpm per cell) were added to Wheaton glass vials, or to Wheaton glass vials first incubated with Medium A at 37° for 24 hr. These vials were incubated at 37° and at 6° for the times indicated, and subsequently washed and prepared for scintillation counting as described in *Methods*. ●, incubation in untreated vials at 37°; ■, incubation in untreated vials at 6°; ○, incubation in pretreated vials at 37°; □, incubation in pretreated vials at 6°. The average and range of triplicate measurements are given. (*B*) Effect of the cell density in the monolayer. Single-cell suspensions at different densities were placed in Wheaton glass vials, in Medium A, and the cells were permitted to grow for 24 hr as described in the text. At this time, the monolayers were fully confluent (●), one-half fluent (□), or one-quarter confluent (△), based on the relative areas of cell surface and exposed glass. The standard assay was then conducted with 1-ml aliquots (40,000 cells per ml; 1.2 cpm per cell) of sBHK cells labeled with [^{3}H]leucine. If the single cells were attaching to submicroscopic gaps in the monolayer, we would expect that they would attach to the substratum even after removal of the monolayer cells. Monolayers were therefore prepared in Wheaton glass vials by incubating 2×10^6 sBHK cells for 24 hr in Medium A. The layers were then dispersed with 0.005% crystalline trypsin in phosphate buffered saline (pH 7.4), and removed from the vials by aspiration after addition of 2 molar equivalents of soybean trypsin inhibitor. The vials were then incubated with 1-ml aliquots of single cells (40,000 cells per ml; 1.2 cpm per cell) under standard assay conditions. No adhesion was observed (○- - -○). All measurements were made in triplicate, and the range and average of each set is shown. Washing procedures and liquid scintillation counting were conducted as described in the *text*.

Adhesion Assay. The assay measures the adhesion of single cells to a cell monolayer. The cell monolayers were attached to the bottoms of Wheaton liquid scintillation vials (3001-1A) or to Linbro multiple-well tissue culture dishes. The choice of glass or plastic was determined by the cell type; some monolayers adhered more firmly to one than to the other. BHK

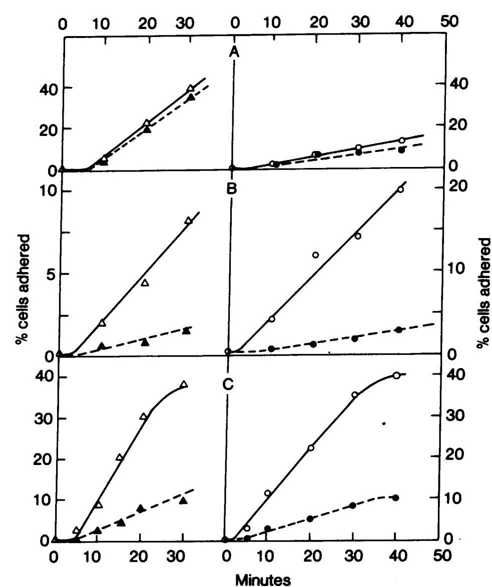

FIG. 4. Kinetics of cell adhesion to homologous and heterologous monolayers. Monolayers were prepared in Linbro tissue culture wells and assays were conducted at 37°. The following single-cell suspensions labeled with [^{3}H]leucine were used at the indicated densities (cells per ml): (*A*) sBHK, 30,000; mouse teratoma, 30,000; (*B*) mouse teratoma, 30,000; mouse-kidney fibroblasts, 30,000; (*C*) chick embryonic neural retina, 100,000; chick embryonic heart, 100,000. *Open symbols* designate the interaction of single cells with the corresponding homologous monolayer, and *closed symbols* with the heterologous monolayer. (*A, left*) sBHK single-cell suspension with sBHK monolayer [△——△] or teratoma monolayer [▲——▲]; (*A, right*) teratoma single-cell suspension with teratoma monolayer [○——○] or sBHK monolayer [●——●]. (*B, left*) teratoma single-cell suspension with teratoma monolayer [△——△] or with mouse-kidney monolayer [▲——▲]; (*B, right*) mouse-kidney single-cell suspension with mouse-kidney monolayer [○——○] for teratoma monolayer [●——●]. (*C, left*) neural retina single-cell suspension with neural retina monolayer [△——△] or heart monolayer [▲——▲]; (*C, right*) heart single-cell suspensions with heart monolayer [○——○] or neural retina monolayer [●——●]. The results are from a typical experiment, and the points are averages of duplicate determinations. The variation between duplicate points was less than 15% in each experiment. In six experiments, neural retina cells adhered from 3.5- to 12-fold more rapidly to neural retina (homologous) monolayers compared to heart cells adhering to neural retina (heterologous) monolayers (average 5.6).

TABLE 1. *Adhesive rate constants of various cell types*

Cell type	ARC	Single-cell concentration range (cells/ml)
sBHK	1.4 ± 0.3 (18)*	10^3–10^6
nBHK	0.7 ± 0.14 (3)	2 × 10^4–2 × 10^5
Teratoma	0.25 ± 0.08 (6)	3 × 10^3–2 × 10^5
3T3	0.22 ± 0.05 (8)	10^4–2 × 10^5
SV3T3†	1.1 ± 0.3 (7)	10^4–1.2 × 10^5

The adhesive rate constant (*ARC*) is measured in the linear part of the cell adhesion curve and is defined as the percentage of single cells in the suspension that adhered per min to the monolayer. All cell types were tested with a monolayer of the same cell type and 0.25 ml of single-cell suspension per cm² of monolayer. The sBHK monolayers were grown in glass vials; the other monolayers were grown in Linbro tissue culture dishes. Growth conditions are detailed in *Methods*.

* Numbers in parentheses represent the number of experiments with different preparations of cells and monolayers.

† For SV3T3 cells grown in Dulbecco medium rather than BHK medium, the ARC is 1.8 ± 0.4. 3T3 cells grown in either Dulbecco or BHK medium have the same ARC. SV3T3 cells grown for 4 months in BHK medium showed a stepwise declining ARC, which eventually stabilized at 0.4.

cells adhered to both substrata and formed monolayers with identical adhesive properties.

Monolayers were prepared by addition of single-cell suspensions to the glass or plastic vessels in Medium A. Monolayers of neural retina and liver cells were prepared from suspensions of single cells containing small cell aggregates (5–10 cells each). Because of the necessity for completely covering the substrata, cells were seeded at densities that resulted in stacking of the cells an average of 2–3 cells thick for sBHK (see Fig. 2) and SV3T3 or partial overlapping (i.e., 3T3, heart). The suspensions, prepared as described above, contained the following numbers of cells per cm² of substratum: nBHK and sBHK, 3 × 10^5; SV3T3, 4 × 10^5; MKA, 1.6 × 10^5; 3T3, 1 × 10^5; heart and teratoma, 2.4 × 10^5; and neural retina, 3 × 10^6. In order to form stable monolayers, the tissue-culture cell lines were maintained under these conditions for 24 hr, and the embryonic cells for 48 hr. All monolayers were inspected microscopically for confluency before use, washed twice with serum-free Medium B, and overlaid with this medium for about 20 min at 37° before adhesion experiments.

The adhesion assay was initiated by removal of Medium B from the monolayer (by aspiration) and its immediate replacement by the labeled single-cell suspension such that the ratio of cell suspension to area of the monolayer was equal to or less than 0.4 ml per cm² (see below). The mixture was shaken in a Warner–Chilcott reciprocal waterbath shaker at the specified temperature and at 60 strokes per min (8 cm per stroke); the rate of adhesion was constant between 30 and 100 strokes per min, above which it declined.

After incubation, the cells remaining in suspension were removed from the monolayer by aspiration and subsequent washing three times at the temperature used for the incubation. The wash solution (Medium B without amino acids, about 0.5 ml/cm² of monolayer) was carefully introduced against the side walls of the vessels, and removed by gentle aspiration with a Teflon capillary tube held against the wall. Three washes required about 10 sec.

The monolayers were then lysed with 1.5 ml of 1.0 M NH_4OH (three washes, 0.5 ml each, for monolayers on Linbro plates), which solubilized 99% of the radioactivity, and the combined extracts were counted in 15 ml of toluene–Triton X-100 (2:1) scintillation fluid containing 8.25 g of PPO and 188 mg of dimethyl-POPOP per liter of toluene. Counting efficiency was about 30%; the NH_4OH quenched about 5%. In the experiments in Fig. 1, 6 ml of H_2O were used to lyse the monolayers in the Wheaton vials and the suspensions were counted in the vials after addition of 15 ml of the liquid scintillation fluid.

RESULTS

Kinetics of Cell Adhesion. Fig. 1 shows a typical progress curve of the accumulation of radioactivity by an sBHK monolayer exposed to labeled single sBHK cells. The fact that only whole cells were attached is shown by the equivalent disappearance of cells from the suspension, and the observation of the attachment of single cells to the monolayer (see Fig. 2). The adhesive process is markedly temperature dependent, and is abolished at 6°. The progress curves of attachment typically showed about 5-min lag periods at 37°, followed by 20–40 min where the rate of adhesion was essentially constant, allowing the definition of an adhesive rate constant. The adhesive rate constant is characteristic of each cell type (Table 1). Under standard conditions, where sedimentation was not rate-limiting, the adhesive rates were directly proportional to cell number, rather than cell density, over a 1000-fold range (Table 1). This fact contributes to the decline in rate with time (see legend to Fig. 1). It is noteworthy that the rate of adhesion with different preparations of the same cell type showed little variation over several months (Table 1); 18 such preparations of sBHK cells were used.

Site of Cell Attachment. A critical question was whether the single cells adhered to the cells in the monolayer, or to the substratum (glass, plastic). The following results show that the single cells adhered to the cells (or intercellular material) in the monolayer.

(*a*) Microscopic examination indicated that cells from the suspension that attached to the monolayer were round, lying on top of the monolayer, and readily distinguishable from the typical fibroblast appearance of the cells in the original monolayer (Fig. 2*A* and *B*). These results were confirmed by autoradiography, where >98% of the round, but none of the fibroblast-type, cells were found to be labeled (Fig. 2*D*). Further, even at the highest cell densities used in the standard assay, and where the labeled cells were often contiguous after attaching to the monolayer, no labeled cell aggregates were detected.

(*b*) Under standard assay conditions the labeled single cells rapidly adhered to glass (or plastic), but the kinetic properties were different from those observed with the confluent monolayers (Fig. 3*A*). The cell–glass reaction showed no lag period, and a much more rapid initial rate. Surprisingly, however, adhesion to the glass essentially stopped, although the suspension still contained 75% of the single cells. Adhesion kinetics with *subconfluent* monolayers (Fig. 3*B*) resembled the kinetics obtained with the confluent monolayer or with glass, depending on the degree of subconfluency.

(*c*) The temperature dependence of cell–monolayer and cell–glass adhesive kinetics was markedly different (Fig. 3*A*).

(*d*) The labeled single cells in suspension did not "exchange" with the unlabeled cells in the monolayer (see also Fig. 1).

(*e*) If single cells attached to the monolayer by binding to the substratum through submicroscopic gaps between the cells, then adhesive specificity should not be observed (see below). Furthermore, when sBHK monolayers were grown either on glass in Medium A and removed with trypsin, or on plastic in serum-free Medium A and removed by washing with phosphate buffered saline, labeled single cells subsequently did not attach to these substrata (Fig. 3*B*). If the substrata were cleaned with acid or alkali, the single cells attached at normal rates. Thus it appears that the monolayers may produce substances that inhibit the adhesion of single cells to the substratum. Such substances do not affect adhesion to one-quarter confluent monolayers, but could possibly account for the lower adhesion to half-confluent monolayers (Fig. 3*B*). It should be noted that small quantities of denatured serum proteins markedly inhibit the adhesion of cells to glass but show no effect on the rate of attachment of cells to monolayers (J. Umbreit, unpublished observations).

Adhesive Specificity. The assay method described above was designed to differentiate between and to quantitate specific and nonspecific intercellular adhesion. The results with BHK cells were typical of those attained with many different cell types tested. In further studies, parallel experiments were conducted simultaneously, in which monolayers of cell type A were exposed to single-cell suspensions of cell type A (homologous) or type B (heterologous) and vice versa (Fig. 4). Specific adhesion is operationally defined as a markedly higher adhesion rate of cells to homologous compared with heterologous monolayers.

The established fibroblast cell lines BHK, polyoma-transformed BHK, and 3T3 gave single-cell suspensions that showed little or no adhesive specificity (Fig. 4*A*); they attached at essentially the same rates to homologous monolayers as to monolayers of heterologous cell lines.

Embryonic chicken neural retina and heart cells as well as secondary cultures of mouse kidney and teratoma cells represent pairs of cells exhibiting adhesive specificity (Fig. 4*B* and *C*). Adhesive specificity can be displayed by cell type A against B but not against cell type C. This phenomenon is shown by the teratoma cell suspensions, when the results with homologous monolayers and mouse kidney monolayers (specific) and with sBHK monolayers (nonspecific) are compared. Similarly, while chick embryonic neural retina cells exhibit specificity when tested both with chick embryonic (parenchymal) liver *and* heart cells, little specificity is exhibited when liver cells and heart cells are tested with each other.

Effect of Trypsin Treatment. A potential anomaly may be inherent in the assay method. Cells in the monolayers were permitted 24–48 hr to "repair" after trypsin treatment, whereas the single cells were trypsinized just before use. The mode of single-cell preparation is possibly crucial to the adhesive properties of the various cells. Nonspecific adhesion may thus result from the destruction of specific sites on the plasma membrane, whereas specific adhesive sites may be uncovered by the action of trypsin on the glycocalyx. It is also possible that cells that show specific adhesion have retained only a fraction of their specific sites. However, the assay described above should be useful in studying these potential anomalies. Preliminary experiments have already shown that the adhesive properties of various cells are markedly different when the cells are exposed to trypsin and are given time to "repair."

DISCUSSION

This paper describes a method for measurement of intercellular adhesion that appears to have significant advantages over other presently available methods. The assay is rapid and reproducible, and requires only a small number of cells for each experiment. Adhesive studies have been conducted with a wide variety of cell types, including embryonic cells and established cell lines of neural, epithelial, and fibroblastic morphology.

The present studies establish that freshly dissociated cells exhibit specific adhesive properties when tested with "recovered" cell monolayers, without the need for prolonged recovery of the dissociated cells. In further experiments it has been shown that the activity of model multivalent adhesive factors such as concanavalin A can be measured by this assay (9), forming the basis for quantitative measurements of lectin agglutination. Current studies, using this assay method, are directed at isolating the cell-surface components responsible for specific and nonspecific intercellular adhesion.

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